

# Can Dietary Pomegranate Peels Reduce Stress Responses Associated with Group Mixing of Holstein Beef Calves?

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**Abstract:** The study investigated whether dietary CPE (concentrated pomegranate peel extract) may mitigate the negative effects associated with group mixing prior to marketing and enhance beef meat quality. Twenty-two bull calves were reared in triplets and divided to control ( $n = 9$ ) and CPE-treated ( $n = 13$ ) groups. CPE was supplemented during eight months of rearing, and calves were mixed 34 d prior to marketing to control and treatment groups. Calves were monitored for weight gain, rumination, activity, metabolic and oxidative stress responses. Finally, meat quality traits were examined. The results find that pre-mixing activity ( $P < 0.0001$ ), non-esterified fatty acids ( $P = 0.02$ ) and plasma testosterone ( $P = 0.005$ ) levels were higher in the treatment group. Following mixing, activity ( $P < 0.0001$ ) and plasma anti-oxidative capacity ( $P = 0.05$ ) increased in the treatment compared to the control group. In spite of the above, dietary supplementation of CPE didn't reveal improvement of meat quality parameters by means of meat pH and shelf life. It indicated that improved serum anti-oxidant capacity in the CPE calves was not sufficient to prevent the mixing effects on meat quality. A combined CPE concentration and mixing management should be examined in order to reduce mixing related effects on meat quality.

**Key words:** Bull calves, group-mixing, CPE, anti-oxidative capacity, non-esterified fatty acid, testosterone.

## 1. Introduction

Animal handling practices prior to marketing are a growing concern in many countries around the world, as they have impact on animal welfare [1]. At the course of handling, animals are exposed to physical and psychological stresses, such as the breakdown of social groupings and mixing with unfamiliar animals [2].

Mixing of unfamiliar bulls causes injuries, bruises and body weight losses [3], leading to significant economical outcome [4].

Lawrie and Ledward (2006) [5] reported that with higher levels of stress poorer meat quality is eminent.

Pre-slaughter handling can affect both carcass and meat quality. The major influence of pre-slaughter handling on lean meat quality is through the potential effect on muscle glycogen stores. If these stores are depleted by chronic stress, the extent of postmortem acidification is reduced, leading to the production of DCB (dark cutting beef), which is prone to spoilage and has poor organoleptic qualities. The major cause of DCB is mixing unfamiliar animals, thus promoting agonistic behavior, particularly in young bulls. Therefore pre-slaughter handling practices which encourage mixing increase the incidence of DCB [6], leading to harmful economic implications.

However, the mixing policy is an integral part of beef cattle production. So, one should seek solutions to

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reduce the deleterious impact of mixing unfamiliar individuals. Since oxidative stress is the outcome of a line of behavioral and physiological responses, a notion we intended to explore was whether long-term supplementation of dietary antioxidants might reduce the oxidative stress effects induced by dramatic event such as group mixing.

Natural antioxidants can protect the cellular components from oxidation processes caused by reactive oxygen species [7], including those which occur during and after the slaughter. Lipid oxidation in the meat results in the formation of free radicals, which may lead to the oxidation of meat pigments and to the generation of rancid odors and flavors [8]. Dietary supplementation of vitamin E, a potent lipophilic anti-oxidant, may for instance, slows down lipid oxidation, and hence expand the shelf-life of the meat [9] and improve lipid stability and meat color [10].

Several phenolic compounds such as gallic acid, cyanidin, quercetin and catechin were also found to have a high antioxidant activity [11]. These phytochemicals, together with punicalagins, ellagic acid and others, are the principal anti-oxidants in the pomegranate fruit [12]. The antioxidant characteristics of all parts of the pomegranate fruit, including the peel, were proven in several studies [13, 14]. Pomegranate was already tested as a potential beneficial additive for enhancing meat quality. It was found that adding pomegranate components to goat [15] and chicken patties [16] had some positive effects of reducing meat lipid peroxidation.

Based on pioneer studies on pomegranate peel dietary supplementation [17], a commercial by-product, namely, CPE (concentrated pomegranate peel extract) has recently been developed. This additive was already found to induce various productive and health-promoting effects [17-19]. However, to the best of our knowledge, the potential of CPE to attenuate oxidative stress damages related to mixing of beef cattle, including effects on meat quality traits, haven't been tested yet.

## 2. Materials and Methods

### 2.1. Calves' Husbandry and Nutrition

Twenty-two young Holstein bull horned calves, not castrated, were included in this experiment. They were randomly divided into two groups: control (C) ( $n = 9$ , BW =  $281.5 \pm 93$ , age  $7.2 \pm 0.1$  months) and treatment (T) ( $n = 13$ , BW =  $295.5 \pm 82$ , age  $7.5 \pm 0.07$  months) (BW and age;  $P > 0.05$ ). The calves were housed in groups of three in  $9 \text{ m}^2$  pens.

Both groups were reared in the same paddock and fed TMR (total mixed ration) (Table 1). Weight measurements were performed monthly, 1 DBM (day before mixing), 3 and 33 DPM (days post mixing).

Calves in the T group received the CPE supplementation between the ages of  $7.3 \pm 0.07$  and  $15.7 \pm 0.07$  months. The CPE was initially poured over the TMR at a concentration of 3%, on DM (dry matter)

**Table 1 Ingredient and chemical composition of the TMR (total mixed ration) fed to experiment calves.**

Components (g/kg of DM)	Amount
<b>Ingredients composition</b>	
Barley grain	402
Ground maize grain	264
Soybean meal (solvent-extracted)	27.06
Wheat barn	51.6
Gluten feed	35.8
Vetch hay (Vicia)	81
Wheat silage	89
Broiler liter silage	33
Minerals and vitamins	2.56
NaCL	3.8
Limestone	10.15
<b>Chemical components</b>	
ME, MJ/kg of DM	2.83
CP, g/kg of DM	132
Organic matter, g/kg of DM	950
NDF, g/kg of DM	240
Ether extract, g/kg of DM	28
Soluble carbohydrates (g/kg)	461
Roughage (g/kg)	170
Ash (g/kg)	50
Ca (g/kg)	8
P (g/kg)	4.68
Vitamin A (IU)	7000

basis, for three months, thereafter concentration was increased to 4%, as shown in Fig. 1. The CPE of the “wonderful” cultivar was supplied by Gan Shmuel Food Ltd. (Gan-Shmuel, Israel). It was made by chopping up the pomegranate parts remaining after pomegranate juice production, including peels and residual arils, followed by extraction in water, filtering, evaporation procedures and pasteurization [18]. The final extract was standardized to ensure uniform and constant DM content [17, 18].

The CPE composition used in this experiment (in g/kg of DM) was: soluble sugars (288), crude protein (20.8), ash (50.2), total soluble phenolics (63), punicalagins (26.5), ellagic acid (2.5); the pH of the extract was 2.65 [19].

Thirty four days before marketing the calves were moved to two paddocks, as requested by the abattoir, one for the C and the other for the T group. The calves were finally weighed on the marketing date, at a mean age of  $523 \pm 0.34$  days and sent to a commercial abattoir.

## 2.2. Rumination and Activity Measurements

In order to examine the behavioral changes associated with group mixing, all calves were equipped with rumination tags (Hi-Tag™, SCR Engineers, Netanya, Israel) that measured the duration of daily rumination in minutes (min/day). The rumination tags

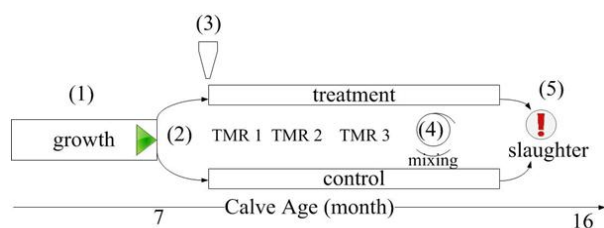
were hung on the neck of each calf during the entire trial period. The rumination data were logged in 2h blocks transferred to the computer and analyzed using Matlab software (Matlab 6.1, MathWorks, Natick, Massachusetts, USA). Activity evaluation included the total number of steps, number of rest-bouts (number of times a calf lies down and stands up again) and rest-time (duration of rest in minutes). These data were recorded by a behavior sensor tag (Pedometer Plus™, AfiFarm® Dairy Herd Management software) that was fitted to the forelimb of each calf. Daily activity is presented for 24 h period, day-time activity was considered as the activity during light hours, hence from 04:00 am until 18:00 pm, and night-time activity was defined as the period between 18:00 pm and 04:00 am. The variables that were collected were analyzed in the following form: sum of daily (24 h), day-time and night-time number of steps, sum of daily, day-time and night-time lying down time in minutes, and sum of the number of daily, day-time and night-time rest-bouts. Average values for every parameter were obtained for each group for the different periods: 20 DBM (days before mixing), 24 h PM (post-mixing), 2, 3-14 and 15-25 DPM.

## 2.3 Blood Sampling

Blood was sampled 1 DBM, 1 and 3 DPM from the caudal vein, using EDTA-containing tubes, heparinized tubes and serum clot activator tubes. The blood was centrifuged at 1,500 g at 4 °C to separate the cells from plasma and to collect serum. These samples were frozen in liquid nitrogen and kept at -20 °C until use.

## 2.4 Determination of Serum Anti-oxidant Capacity

Two methods were used to evaluate total antioxidant capacity, the FRAP (ferric reducing antioxidant power) assay [20] and the Chemiluminescence-inducing cocktail method [21, 22]. The FRAP assay uses antioxidants as reluctant in a redox-linked colorimetric method, employing an



**Fig. 1** Experiment schedule.

(1) Growth period of calves since parturition; (2) Separation of 22 calves into control and treatment calves, reared in triplets; (3) CPE was poured on the ration up to the end of experiment; (4) Calves were mixed (from triplets in a pen) into control ( $n = 9$ ) and treatment ( $n = 13$ ) groups; (5) All calves were sent to slaughter in organic groups. TMR—total mixed ration in first, second and third periods of experiment fed to all calves, TMR 1, 2, and 3 slightly differ in feed composition.

easily reduced oxidant system. Analyses were performed in duplicates on 1 DBM, 1 and 3 DPM heparinized plasma samples, and data were presented as  $\mu\text{M}$  ascorbic acid equivalents.

For the Chemiluminescence-inducing cocktail method we used serum samples. This method is based on generation of light-conjugated free radicals production. The anti-oxidant capacity of a sample is evaluated by its potential to quench the light generated by the system. Thus, lower LDCL (luminol-dependent Chemiluminescence) values reflect samples with higher anti-oxidant capacity. The reaction cocktail was comprised of Hank's buffer (cat #02-016-1A, BI),  $\text{H}_2\text{O}_2$  and 1 mM of cobalt chlorine, sodium selenite, and luminol. A volume of 20  $\mu\text{L}$  serum was added to the reaction mixture and analyzed immediately in a Lumac type 2500 M luminometer for LDCL generation. The samples were measured during 6.5 min and values were calculated as the sum counts of the entire measurement period.

### 2.5 Analysis of Plasma NEFA (Non-esterified Fatty Acids)

We used a two reaction, enzymatic-based assay that was adapted and validated to quantify NEFA in bovine heparinized plasma, using micro-titer plates [23]. The analysis was performed in duplicates samples on 1 DBM, 1 and 3 DPM using the commercial kit NEFA-HR (2) R1 set, (cat #. 434-91795 Wako chemicals GmbH).

### 2.6 Determination of Plasma Testosterone Concentrations

In order to discover whether the dietary CPE influenced the testosterone levels, and hence the calves' behavior, we sampled blood from the caudal vein 3 months and 1 day before mixing into EDTA-containing tubes. Testosterone levels were measured in duplicates using a DRG Elisa kit (ELISA EIA-1559, DRG Instruments, GmbH, Marburg, Germany), according to the manufacturer's instructions.

### 2.7 Meat Quality Analyses

The calves were slaughtered at a commercial abattoir one day after arrival. The pH of m. LTL (longissimus thoracis et lumborum) was measured using a glass electrode 24 h after the carcasses were chilled.

At slaughter, the pelvic, kidney, testicles and heart fat depots were weighed. The quarters of the carcasses were chilled overnight in a refrigerated room, followed by dismantling of the carcasses. Slices of about 250 g from the M. longissimus dorsi between the 12th and 13th ribs were sampled from the carcass of each calf. These samples were kept in vacuum bags at  $-20^\circ\text{C}$ . These slices were used for analyses of DM, ash, water content, crude protein, ether extract, fatty acid profile and lipid oxidation. To this end, 5 g ground meat samples were dried at  $105^\circ\text{C}$  for 24 h [24]. Determination of ether extract was carried out by the Soxhlett method [25], and FAME (fatty acid methyl esters) was performed as previously described [26] with some modifications [27, 28]. The extent of meat lipid oxidation was determined in duplicates by the formation of TBARS (thiobarbituric acid-reactive substances) as previously described [29, 30] with some modifications.

Meat samples were tested for TBARS values one day (D1), 4 days (D4), 9 days (D9), 16 days (D16) and 31 days (D31) after thawing. The results are calculated [31] and expressed as milligrams malondialdehyde/kg meat.

### 2.8 Statistical Analysis

All means are presented  $\pm\text{SEM}$  and analyzed with the use of SPSS for Windows (version 17.0). The differences of weight gain and meat analyses were analyzed using the 2-way ANOVA test. For plasma NEFA levels, testosterone levels, fatty acid profile and fat percentages in meat; we used an independent one-tailed student t-test. The differences in activity, rumination, FRAP, and anti-oxidative activity analysis among groups was tested using a repeated

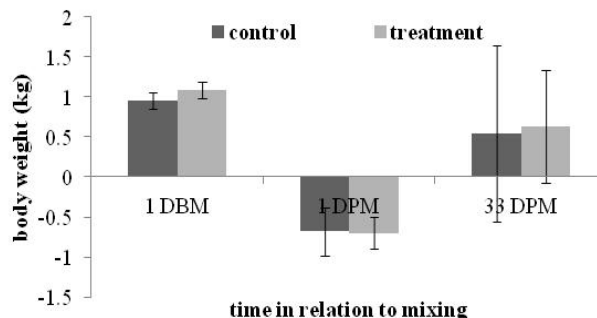
measurement ANOVA (analysis of variance). The meat analyses differences were tested using the one-way ANOVA test. Comparison of meat pH distribution was tested using Chi-square test. We used the Spearman correlation coefficient ( $r$ ) to test the one-tailed non-linear correlation between variables. Statistical significance was declared at a probability level of  $P \leq 0.05$ .

### 3. Results

#### 3.1 Mixing Effect on Weight Gain, Plasma Levels of NEFA and Testosterone Concentrations

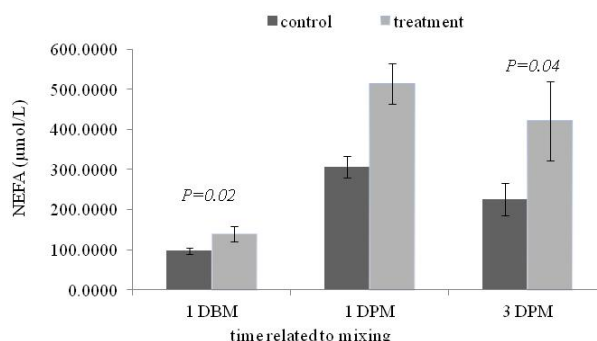
The group mixing had a significant impact on the animals weight gain with no difference between the C and T groups (pooled groups effect;  $P = 0.04$ , group  $\times$  mixing days;  $P = 0.9$ ; Fig. 2). Average weight loss of calves was  $6.1 \pm 3$  and  $6.3 \pm 2$  kg for the C and T groups, respectively. Moreover, even 33 DPM, the average weight gain did not return to the values obtained before mixing. Additionally, one calf suffered from a broken leg on the day following mixing and needed to be excluded from the experiment.

In response to mixing, plasma NEFA levels increased significantly for pooled groups ( $P < 0.0001$ ). However, NEFA concentrations were statistically higher for T over C group in all sampled days (Fig. 3). Additionally, plasma testosterone concentrations were significantly higher in the T group, 3 months and 1 day before mixing (Fig. 4). A positive correlation was found between testosterone levels three months DBM and weight gain 1 DBM ( $r_s = 49$ ,  $P = 0.02$ ,  $n = 18$ ). Additionally, testosterone levels three months before mixing was positively correlated to testosterone concentration 1 DBM ( $r_s = 54$ ,  $P = 0.01$ ,  $n = 18$ ), NEFA levels 1 DPM ( $r_s = 45$ ,  $P = 0.02$ ,  $n = 19$ ), number of steps 20 DBM ( $r_s = 44$ ,  $P = 0.04$ ,  $n = 17$ ), steps 1 ( $r_s = 44$ ,  $P = 0.03$ ,  $n = 18$ ) and 3 DPM ( $r_s = 43$ ,  $P = 0.03$ ,  $n = 18$ ). The testosterone levels 1 DBM were also positively correlated to testosterone levels three months earlier ( $r_s = 54$ ,  $P = 0.01$ ,  $n = 18$ ), NEFA levels 1 DPM ( $r_s = 62$ ,  $P = 0.003$ ,  $n = 18$ ), and number of



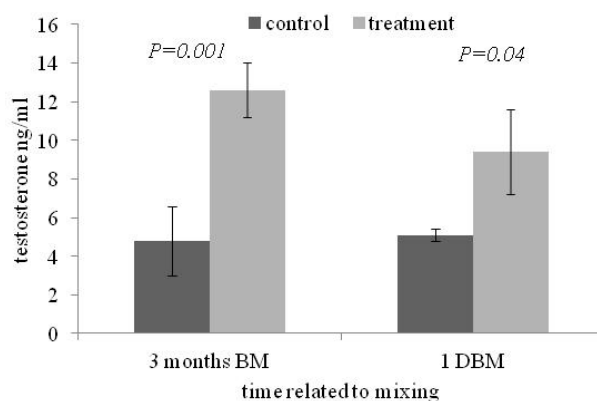
**Fig. 2** The effect of group mixing on weight gain of Holstein bull calves.

1 DBM—1 day before mixing, 3 and 33 DPM—days post mixing. The mixing had a significant effect on pooled groups ( $P = 0.04$ ), however, no mixing  $\times$  treatment interactions were revealed ( $P = 0.9$ ). Data are presented as means  $\pm$  SEM.



**Fig. 3** The effect of dietary CPE on plasma NEFA levels following mixing.

Samples were taken 1 DBM (one day before mixing), one day and three DPM (days post mixing). Data are presented as means  $\pm$  SEM. Significance refers to the difference between groups on each tested day. CPE—concentrated pomegranate peel extract. NEFA—non esterified fatty acids.



**Fig. 4** The effect of CPE on plasma testosterone concentrations in Holstein bull calves.

3 months BM—three months before mixing, 1 DBM—one day post mixing. Data are presented as means  $\pm$  SEM and significance refers to t-test comparison between control and treatment groups. CPE—concentrated pomegranate peel extract.

steps 1 ( $rs = 66$ ,  $P = 0.002$ ,  $n = 17$ ) and 3 DPM ( $rs = 45$ ,  $P = 0.03$ ,  $n = 17$ ). In addition, testosterone levels 3 months before mixing negatively correlated to the rest-time 3 DPM ( $rs = -56$ ,  $P = 0.009$ ,  $n = 17$ ). Finally, the number of steps 20 DBM and NEFA levels 1 DPM were positively correlated ( $rs = 0.59$ ,  $P = 0.006$ ,  $n = 17$ ).

### 3.2 The Effect of Mixing on Plasma Antioxidant Capacity

The total antioxidant capacity evaluated by the FRAP method was not affected by mixing. The values were  $181.6 \pm 15$  and  $189.5 \pm 13$  on 1 DBM,  $173.6 \pm 19$  and  $178.1 \pm 21$  on 1 DPM, and  $210.7 \pm 24$  and  $198.3 \pm 20$   $\mu\text{M}$  ascorbic acid equivalent on 3 DPM, for the C and T groups, respectively ( $P > 0.05$ ). However, the anti-oxidative capacity of the serum measured by the chemiluminesce method revealed an improved antioxidant capacity for the T group (Fig. 5). The anti-oxidant capacity of the T group was significantly superior relative to the C group only 1 DPM, as judged by the lower LDCL values in the different days. However, when we tested only the effect of the treatment on LDCL, without considering the day factor, the results were significant ( $P = 0.04$ ). Furthermore, there was no interaction between treatment and days in relation to mixing ( $P = 0.8$ ).

### 3.3 Activity and Rumination Following Group Mixing

The mixing had a significant effect on the calves' activity pooled groups: the daily steps in the 20 DBM, 24 h, 2, 3-14 and 15-25 DPM was  $3,508 \pm 445$ ,  $10,305 \pm 783$ ,  $4,062 \pm 638$ ,  $4,913 \pm 657$  and  $6,391 \pm 1,167$  steps/d, respectively ( $P < 0.0001$ ). Also the daily number of rest-bouts was affected by the group mixing, being  $19.1 \pm 0.8$ ,  $10.4 \pm 0.7$ ,  $16.4 \pm 1.1$ ,  $17 \pm 0.5$  and  $19.7 \pm 0.9$  rest-bouts/d, respectively ( $P < 0.0001$ ), for 20 DBM, 24 h, 2, 3-14 and 15-25 DPM. Daily rest-time differed significantly between the periods around mixing: the calves rested on 20 DBM, 24 h, 2, 3-14 and 15-25 DPM a sum of  $702 \pm 12$ ,  $384 \pm 17$ ,  $609 \pm 20$ ,  $580 \pm 12$  and  $701 \pm 19$  min/d, respectively ( $P < 0.0001$ ).

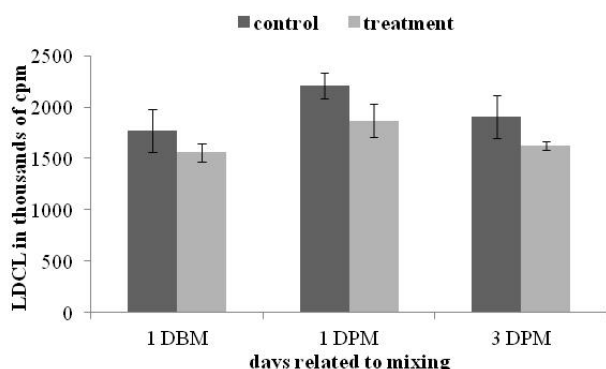
The differences in activity and resting parameters between C and T groups are elaborated in Table 2. It is noticeable that both the daily steps and the day-time steps of the T group are significantly higher than those of the C group, however, during the night-time, the differences in number of steps between the groups were not significant.

The daily sum of rest-time (m/d) in the two groups was not different during the pre-mixing period ( $P = 0.6$ ), nor 24 h post mixing ( $P = 0.8$ ). 24 h-2 DPM, the C group rested longer as compared to the T group ( $P = 0.05$ ), but no differences among the groups were shown

**Table 2** Daily and day-time activity (steps) and rest time in C (control) and T (treatment) groups in relation to mixing.

Period	T/C	Steps		Rest time	
		Daily	DT	Daily	DT
Pre-mixing	C	$2,109 \pm 167$	$1,328 \pm 199$	$703 \pm 19$	$388 \pm 17$
	T	$3,169 \pm 249^{***}$	$1,944 \pm 216^{***}$	$701 \pm 15^n$	$369 \pm 13^n$
24h PM	C	$7,851 \pm 413$	$5,996 \pm 310$	$377 \pm 23$	$152 \pm 11$
	T	$13,395 \pm 702^{***}$	$9,269 \pm 477^{***}$	$391 \pm 28^n$	$182 \pm 17^n$
24h-2 DPM	C	$1,968 \pm 153$	$2,128 \pm 178$	$657 \pm 17$	$352 \pm 16$
	T	$6,129 \pm 574^{***}$	$3,635 \pm 369^{***}$	$561 \pm 30^*$	$299 \pm 22^n$
3-14 DPM	C	$2,700 \pm 145$	$2,623 \pm 344$	$585.5 \pm 10$	$330 \pm 9$
	T	$7,232 \pm 513^{***}$	$5,039 \pm 971^{***}$	$574 \pm 23^n$	$315 \pm 13^n$
15-25 DPM	C	$4,862 \pm 338$	$1,837 \pm 281$	$723 \pm 20$	$450 \pm 15$
	T	$6,891 \pm 2,048^n$	$3,734 \pm 940^{***}$	$678 \pm 27^n$	$384 \pm 16^{**}$

Values are presents as means  $\pm$  SEM. C—control, T—treatment, Daily—24 h period, DT—day time (daily-night time)—between 04:00 am and 18:00 pm. Rest time is measured in minutes per day and day time. Pre-mixing mean—average of the 20 days prior to mixing, PM—post mixing. Numbers indication for two-tail  $P$  value is between treatment pairs in the same categories:  $n$ —not significant,  $^*P \leq 0.05$ ,  $^{**}0.001 < P < 0.01$ ,  $^{***}P < 0.0001$ .



**Fig. 5** The effect of dietary CPE on plasma anti-oxidative capacity of Holstein bull calves.

1 DBM—one day before mixing, 1 and 3 DPM—one and three days post mixing. T-test result of ANOVA repeated measurement revealed an effect of treatment alone ( $P = 0.04$ ), but no interaction between treatment and days in relation to mixing ( $P = 0.8$ ). CPE—concentrated pomegranate peel extract. LDCL—luminol dependent chemiluminesce. Please note that lower LDCL counts represent higher anti-oxidative capacity.

in the 3-14 and 15-25 DPM (Table 2). In addition, the rest at night-time was longer for all calves, with a non-significant tendency of the C group to lie down longer than the T group.

Rest-bouts, which are positively correlated with rest-time [32] decreased 24 h PM and then calves gradually restored their pre-mixing values 15-25 DPM. Daily, day-time and night-time number of rest-bouts were similar in the two groups in all periods. However, the impact of mixing regardless of treatment was clearly noticeable ( $P < 0.0001$ ).

The average daily rumination duration was affected by mixing (the difference between 20 DBM and 24 h, 2, 3-14 and 15-25 DPM were significant regardless of group identity;  $P < 0.0001$ ), however, it did not differ between groups. Prior to mixing, the C and T groups ruminated  $306.6 \pm 24$  and  $330.5 \pm 18$  min/d, respectively ( $P = 0.5$ ). 24 h PM, rumination activity took place for only  $175.8 \pm 17$  and  $157.5 \pm 17.8$  min/d for the C and T groups, respectively ( $P = 0.3$ ); rumination increased back to its initial pre-mixing values 24 h-2 DPM and 3-14 DPM ( $290.8 \pm 12$  and  $295.8 \pm 19$  min/d,  $P = 0.4$ ), for the C and T groups, respectively. Interestingly, during the 15-25 DPM period, rumination differed significantly among groups

( $333.6 \pm 24$  m/d and  $349.8 \pm 18$  m/d for the C and T groups, respectively ( $P = 0.04$ )).

### 3.4 Results of Meat Quality Analyses

There were no differences between groups in the following parameters: DM, ash, water content, protein and fat in meat ( $P > 0.05$ ). The average pH values for all meat samples regardless of treatment were  $6.5 \pm 0.02$ . While meat pH values of the T group were  $6.55 \pm 0.03$ , pH of the C group was  $6.60 \pm 0.03$ . These values are higher in comparison with LTL of other Holstein calves (where 60% of carcasses had pH  $< 6$  as oppose to 21% of carcasses in the current trial) reared, handled and slaughtered under the same conditions ( $P = 0.009$ ). The overall fat percentages dressed out of the net carcass weight did not differ between the groups ( $P > 0.05$ ).

The composition of FAME detected in the meat did not differ among groups. The TBARS values of the C and T groups on D1, D4, D9, D16 and D31 post thawing were similar. The results of the repeated measurements test showed that the rise with time after thawing was significant ( $P < 0.0001$ ); however, there was no effect of the treatment ( $P = 0.8$ ) nor was there an interaction between time and treatment ( $P = 0.9$ ).

## 4. Discussion

### 4.1 Effect of CPE Supplementation on Behavior and Metabolism

NEFA (non-esterified fatty acids) are released from lipid stores and oxidized in the liver as an alternative energy source. Thus, its concentration in the plasma is an indication for lipid mobilization to overcome current demand for energy [33]. The T group had higher circulating levels of NEFA compared to the control group. This could be explained by either direct CPE effects on fat metabolism, androgenic effect of CPE on NEFA concentration, or a combination of both.

Dietary pomegranate peels were shown to modulate carbohydrate and fat metabolism [34]. Indeed, decreased levels of blood triglycerides, cholesterol and

glucose were demonstrated following pomegranate consumption [34, 35]. Various mechanisms of action might explain these effects. Among them one can include metabolic effects such as: (1) suppression of energy intake and inhibition of pancreatic lipase activity, leading to decreased absorption of dietary fat [36], (2) changing the interplay between the metabolic hormones leptin, insulin (decrease of both) and adiponectin (increase) [37], and (3) activation of PPAR $\alpha$ , PPAR $\beta/\delta$  and PPAR $\gamma$  [38, 39], the central modulators of lipid and carbohydrate metabolism. However, dietary CPE could have also influenced calves lipid metabolism through regulation of the androgen hormone testosterone. In the current study, plasma testosterone levels of the T calves were higher than those of the C group. This, in turn, was also reflected in the significantly higher activity (already pre-mixing), and the positive correlation between the testosterone levels 3 months before mixing and activity 1 and 3 DPM, suggest together that CPE might have potentially altered calves temperament and may be used in order to predict stress response to mixing months before it occurs. Also in previous studies pomegranate supplementation was associated with increased plasma testosterone levels [40] and activity [41]. Testosterone administration to non-obese aging men prevented visceral fat accumulation [42].

An androgenic effect attributed by testosterone was previously suggested to cause higher frequency of interactions, aggression and movement [43]. Rebuff -Scrive et al. [44] demonstrated that administration of testosterone led to decrease of abdominal adipose tissue LDL, as well as increased lipolytic responsiveness to norepinephrine. Interestingly, a reciprocal effect of free fatty acids on testosterone metabolism was reported *in vivo* [45]. However, weight gain, fat depots, as well as fat content and composition in the meat did not differ among groups, perhaps since pomegranate effect on fat metabolism is localized to specific fatty tissue such as sub-dermal or abdominal fat [44].

#### 4.2 Effect of Mixing on Behavioral and Physiological Measures

Group mixing was accompanied by a high degree of physical activity, which gradually decreased during the post-mixing period. The social and environmental changes which are associated with the establishment of group social hierarchy [46], were behaviorally characterized by aggressive butting, pushing, frequent mounting and increased vocalization (data not shown). Warriss et al. [47] reported that behavioral interactions and associated physical activity occurring during group mixing had led to a considerable rise in plasma creatine phosphokinase activity and free fatty acid concentration. In the present study we observed a noticeable effect of mixing on activity as judged by the number of increased steps along with a tendency of decreased rest-time. This stressful stimulus was further pronounced by increased plasma NEFA levels and decreased rumination and weight gain that we assume were a result of decreased feed intake. These factors similarly affected both groups with no apparent advantage for the CPE calves. Interestingly, however, the significantly higher activity levels of the T group was not further reflected by an attenuation of their daily rumination time, nor by decreased weight gain, compared with the control. It is thus hypothesized that CPE might have contributed to higher efficient utilization of the diet by the T group.

#### 4.3 Effect of CPE on the Antioxidant Capacity of Serum and Meat

Several studies demonstrated a higher anti-oxidant activity of the pomegranate peels in relation to the juice [48], mainly due to water-soluble polyphenols, anthocyanins and hydrolyzable tannins [49, 50]. Phenolic compounds attain their active anti-oxidant activity through free-radical scavenging activity [51], transition-metal-chelating activity [52] and/or singlet-oxygen quenching capacity [53]. These mechanisms may explain the results obtained herein for the T calves which serum was characterized by



higher anti-oxidative activity, in comparison with control. Recent studies from our research team in dairy cows [18] demonstrated that milk anti-oxidative potential can be improved in response to dietary CPE supplementation. Thus, we hypothesized that similar effects might have also been attained in meat. However, the results shown herein raise the possibility that dietary CPE components are not accumulated in meat, as in the case of milk. It cannot be excluded, however, that the timing of group mixing might have obscured the beneficial contribution of edible CPE on meat anti-oxidative parameters. Indeed, the potential of pomegranate was uncovered in a study which tested the *in vitro* effect of pomegranate on goat meat patties. It was shown that the average TBARS values during refrigerated storage were lower in these tested samples [15]. Similar effects were reported also in the case of chicken patties [16], however, other dietary anti-oxidant did not affect the total anti-oxidant status of the plasma and meat pH [53]. These discrepancies may arise from the different application methods used. The dietary concentration of CPE (4% of DM of TMR) we used during the experiment may have not been sufficient for proper anti-oxidative effect on meat lipid oxidation. Generally, tannins that are abundant in pomegranate peels are known to bypass the rumen and are absorbed in the small intestine [54]. Punicalagin, which is responsible for about half of the total anti-oxidant capacity of the juice [50], is highly bioavailable, since it can be completely absorbed as an intact molecule in the plasma [55]. However, it can also be hydrolyzed in cells to yield ellagic acid [56] and only traces of punicalagin metabolites can be detected in tissues [57, 58]. It was suggested that the poor absorption of ellagic acid may prevent tissues from attaining sufficient concentrations for further effects [59]. Again, it cannot be excluded that the timing of group mixing might have obscured the beneficial contribution of edible CPE. Hence, further research is necessary to ascertain whether feeding CPE at higher levels or perhaps in different forms, may potentially

enhance the quality of fresh meat. This should be considered within a broader management practice which would also include timing of groups mixing.

#### *4.4 Effect of Mixing on Meat pH*

DCB (dark cutting in beef) is a state in which the ultimate pH post mortem measured after 12-48 hours is  $\geq 6$ . This can occur when animals are exposed to chronic or long term stress before slaughtering [60]. The meat pH measurements in the current study are considered higher than recommended (mean  $\pm$  SEM;  $6.5 \pm 0.02$ ), and we assume that this might be related to the mixing that had occurred 34 days pre-slaughter.

Mixing unfamiliar beef bulls shortly or immediately before loading led to difficulties in unloading them from the truck [47] and reduction of meat quality [6, 61]. Evidence in the literature as for the recommended period between group mixing and slaughter are not unequivocal; looking for methods to prevent DCB, Warriss et al. [47] found that, two days were required for unfamiliar young bulls to recover from mixing stress, by means of sufficient replenishment of muscle glycogen stores and  $\text{pH} < 6$ . Similarly, Tennessen et al. [62] observed that 10 days post-mixing, bulls showed very little aggressive behavior. Yet, the process of restoring a new social hierarchy for stranger bulls can take at least 12 weeks [63].

Based on the precise activity data monitored in the present study, calves do not seem to restore their pre-mixing behavior even 25 d post mixing and their pH levels after slaughter were higher than 6, even when slaughter occurred 34 days post mixing. These discrepancies might be explained by differences in breeds, rearing and slaughter practices, however, further investigations are required to explore this issue.

## **5. Conclusions**

Mixing of unfamiliar calves has strong behavioral and physiological implications. This was demonstrated by the increased steps, NEFA levels, anti-oxidative activity and decreased rest, rumination and weight gain.

Speaking of meat quality traits, the consequences of mixing may have further led to improper pH levels of the meat after slaughter. From this point of view, the activity monitoring systems utilized hereby and testosterone levels, may serve as predictive tool for the recovery period needed from mixing to marketing.

Dietary supplementation of 4% CPE during 8 months, before marketing, did not prevent reduction of meat quality traits as judged, at least, by its pH values. On the other hand, CPE was found to increase the calves steps, plasma testosterone levels and anti-oxidative capacity. Apparently, CPE supplementation likely benefits calves coping with a stressful event, however, these beneficial effects might have gone obscured due to improper timing of group mixing, or relative to the distance from slaughter. Yet, further investigations are required in order to find out if a different combination of CPE concentration and mixing management would be expressed in enhancement of meat quality.

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